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MOLECULAR PHYLOGENY AND BIOGEOGRAPHY OF A HIGH MOUNTAIN BIVALVE FAUNA: THE SPHAERIIDAE OF THE TIBETAN PLATEAU

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ABSTRACT

Sphaeriids represent a highly diverse family of small bivalves inhabiting freshwater habitats worldwide. They are recognized for their high dispersal capacity as well as for a high capability to adapt to such extreme environments as high mountain regions over 4,000 m above sea level (a.s.l.). The Tibetan Plateau, located in central Asia, is such a high elevation area. Here, we present the first broad spatial sampling of sphaeriids from the Tibetan Plateau, which allowed us to describe the Recent fauna as well as their biogeographical background. Our study revealed six sphaeriid species inhabiting various habitats on the Tibetan Plateau: *Pisidium nitidum*, *P. stewarti*, *P. subtruncatum*, *P. zugmayeri*, *Pisidium* sp. I and *Musculium kashmirensis*. Four species are newly recorded for this region. Based on phylogenetic analyses using molecular information from three different genes (COI, 16S and 28S) we conclude that the extant diversity of Tibetan Plateau sphaeriids might have been caused by multiple colonization events. Furthermore, the zoogeographic affinities of Tibetan Plateau sphaeriids are mostly related to the Palearctic. Similar patterns have been observed in previous studies of other freshwater molluscan taxa in the area.

Key words: *Pisidium*, *Musculium*, Himalayas, dispersal, molecular phylogeny, colonization.

INTRODUCTION

The family Sphaeriidae comprises small to minute freshwater bivalves with shell lengths ranging from 2.5 to 25 mm (Korniushin, 2006). They are the second most diverse family of freshwater bivalves after the Unionidae, with an estimated number of 227 species (Graf, 2013) in five genera: *Eupera* Bourguignat, 1854; *Byssanodonta* d'Orbigny, 1846; *Sphaerium* Scopoli, 1777; *Musculium* Link, 1807; and *Pisidium* C. Pfeiffer, 1821 (Dreher Mansur & Meier-Brook, 2000; Korniushin & Glaubrecht, 2002). However, phylogenetic relationships among and within the sphaeriid taxa are still largely unresolved (Lee & Ó Foighil, 2003; Korniushin & Glaubrecht, 2006, and references therein; Schultheiss et al., 2008), which results in conflicting classifications especially on the generic level (Graf et al., 2013).

Sphaeriids are currently distributed worldwide (except for Antarctica; Bogan, 2008) and are known for their high capability to adapt to diverse habitats and extreme environments, such as high mountain regions > 4,000 m above sea level (a.s.l.) (Preston, 1909; Prashad, 1933; Bössneck, 2012). Furthermore, sphaeriid species may have a large distribution range (i.e., transcontinental distribution), but can also be restricted to small ranges (Korniushin, 2006).

These characteristics as well as their high passive dispersal capacity (Kappes & Haase, 2012) make the Sphaeriidae suitable model organisms for phylogeographic studies, particularly in species-poor high-elevation regions such as the Tibetan Plateau.

The Tibetan Plateau of central Asia is the largest and highest plateau on earth (mean elevation of ~5,000 m a.s.l.; Royden et al., 2008) and surrounded by high mountain ranges: Qilian and Altun Mountains in the north, Himalaya and Karakorum Mountains in the south and west, and Longmen Mountains in the east (Chang et al., 1989). Approximately 1,600 freshwater and saline lakes greater than 1 km^2 are scattered throughout the plateau (Zheng, 1997). These lakes are typically oligotrophic (Xing et al., 2009), and many of them are part of endorheic drainage basins (Revenga et al., 1998). Besides the lakes, numerous smaller lentic water bodies,

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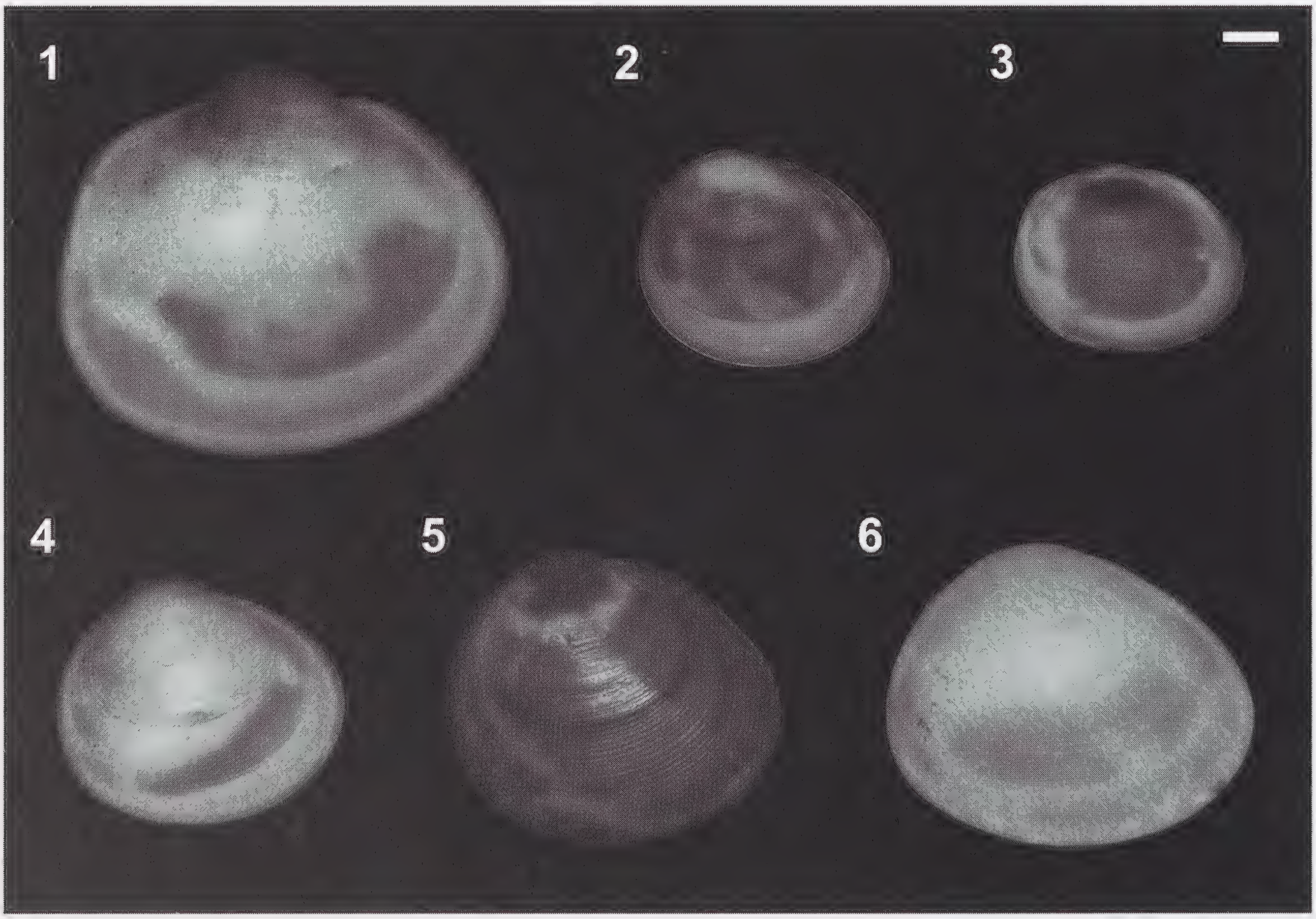
such as ponds and wetlands, are present (An et al., 2009). The plateau with its large glaciers ("third pole"; Xu et al., 2009) is of great importance for regional freshwater systems, because several major rivers of South and East Asia (such as the Yangtze, Mekong, Brahmaputra, and Indus) have their headwaters in this area.

The faunal components of the Tibetan Plateau are mostly thought to be of Palearctic and Oriental origin (Yang et al., 2009), and the freshwater mollusk fauna seems to be mainly characterized by Palearctic species (Oheimb et al., 2011, 2013). Thereby, the Himalayan mountain range forms the southern border to the Oriental region (Cox, 2001; Morrone, 2002) and is assumed to be a major dispersal barrier for freshwater taxa (Bănărescu, 1992; Abell et al., 2008; Oheimb et al., 2013). In addition, extant patterns of diversity and distribution of species throughout the Tibetan Plateau are mainly influenced by extreme geological changes during the plateau's uplift, as well as climate fluctuations especially during the Pleistocene (e.g., Last Glacial Maximum, ca. 20,000 years ago; Yang et al., 2009). Although the Tibetan Plateau was probably not completely covered by an ice sheet throughout the Pleistocene (Zheng & Rutter, 1998; Shi, 2002), the unstable climatic conditions resulted in massive environmental changes over time, such as lake-level fluctuations (Lehmkuhl & Haselein, 2000; Dietze et al., 2010). These changing environments might have triggered diversification processes in Tibetan Plateau freshwater organisms, though the regional freshwater fauna and particularly the mollusk fauna remains species-poor and scarcely studied. A total of three gastropod genera (*Radix* Montfort, 1810; *Gyraulus* Charpentier, 1837; *Valvata* O. F. Müller, 1773) and two genera of bivalves (*Musculium*, Fig. 1; *Pisidium*, Figs. 2–6) were reported for the plateau (Prashad, 1933; Mischke et al., 2010; Bössneck, 2012; Oheimb et al., 2011).

Two recent studies focused on the biogeographical history of pulmonate freshwater gastropods (the lymnaeid genus *Radix* and the planorbid genus *Gyraulus*) on the Tibetan Plateau (Oheimb et al., 2011, 2013).



FIGS. 1–6. Sphaeriid species sampled across the Tibetan Plateau. FIG. 1: *M. kashmirensis* (I), location CN19; FIG. 2: *P. stewarti* (VI), CN07; FIG. 3 *Pisidium* sp. I (II), CN07; FIG. 4: *P. nitidum* (III), CN21; FIG. 5: *P. subtruncatum* (IV), CN14; FIG. 6: *P. zugmayeri* (V), CN22. Roman numerals refer to the respective phylogenetic lineages, see Fig. 12. Scale bar = 0.5 mm.

Although sphaeriid clams often occur in sympatry with *Radix* spp. and *Gyraulus* spp., major biological and ecological differences exist, potentially affecting phylogeographical patterns. First, the clams are soft-substratum-dwelling filter-feeders with a potential to influence ecosystem processes in freshwater systems (Vaughn & Hakenkamp, 2001; Dillon, 2004). Secondly, sphaeriid species show the reproductive strategy of euviviparity, that is, eggs, embryos, and juveniles are bred in specialized structures of the parental gills (Korniushin & Glaubrecht, 2006), providing a comparatively stable environment for the offspring. Thirdly, the (passive) dispersal capacity of sphaeriids is presumed to be very high (Mackie, 1979; Schultheiss et al., 2008), thus playing a major role in colonizing new habitats (Zelaya & Marinone, 2012). In fact, there are several mechanisms enabling these small bivalves to disperse between waterbodies in close proximity, as well as over long distances (Kappes & Haase, 2012). Vector-mediated passive dispersal seems to be of particular importance. For example, clams were found being attached to the limbs of freshwater insects and amphibians (aquatic vectors; Rees, 1965; Bilton, 2001) and to the feathers and legs of different waterbirds, as well as to the extremities of flying insects (aerial vectors; Wesselingh et al., 1999; Figuerola & Green, 2002; Zelaya & Marinone, 2012). Furthermore, Mackie (1979) reported that juvenile sphaeriids are able to survive the gut passage in ducks.

Considering these particular characteristics of Sphaeriidae (in contrast to other Tibetan Plateau freshwater mollusks) as well as the special environmental conditions of the high-elevation Tibetan Plateau, this study has two major aims:

(I) Assessment of the Tibetan Plateau Sphaeriidae fauna (including data about diversity and ecology) based on extensive sampling.

(II) Reconstruction of the biogeographical background of the Sphaeriidae species inhabiting the plateau (e.g., number of colonization events, zoogeographic affinities) based on the phylogenetic information inferred from three different DNA markers.

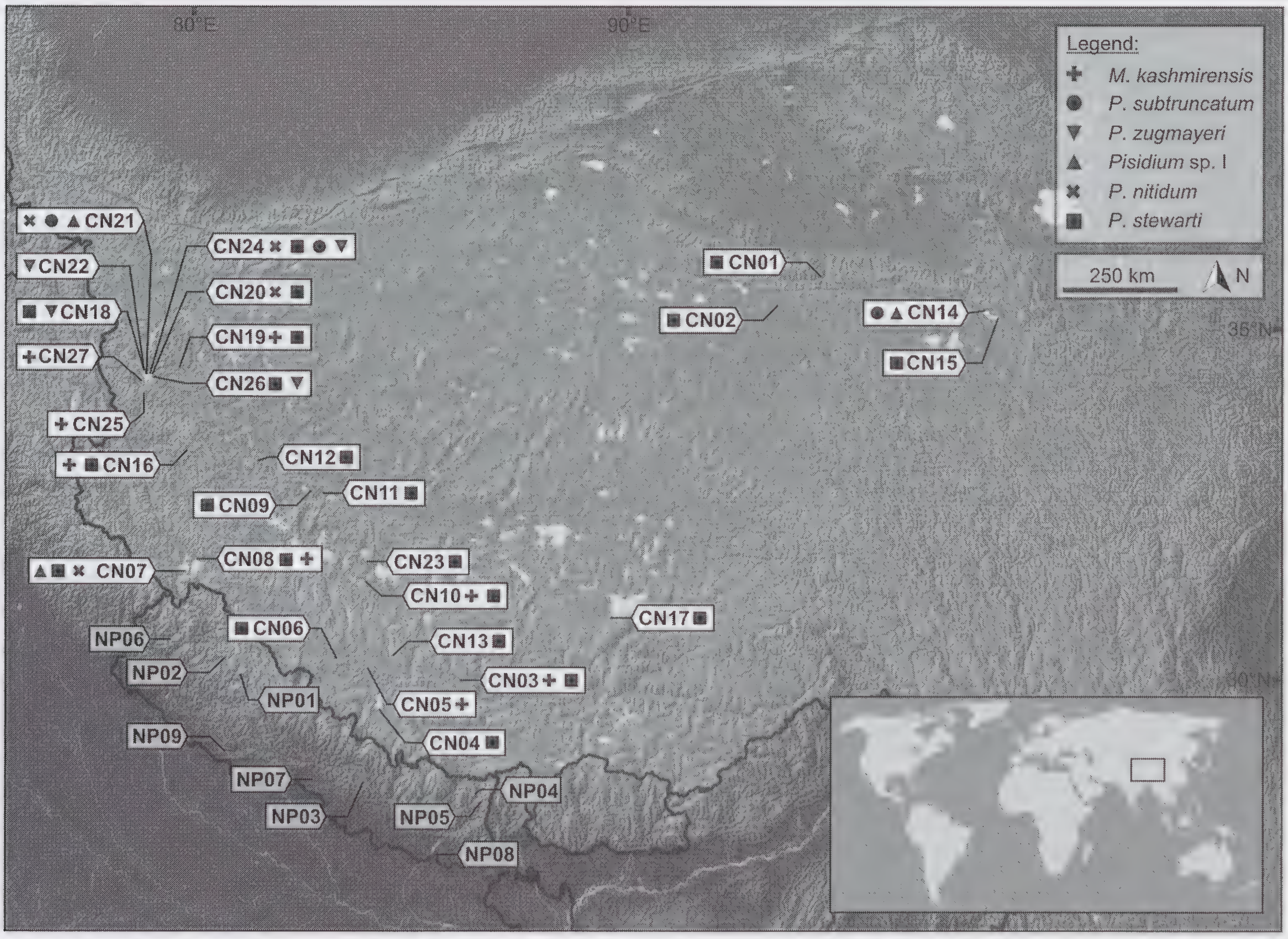


FIG. 7. Sampling sites of Sphaeriidae across the Tibetan Plateau and Nepal. Locality information is given in the Supplementary material (Appendix 1).

MATERIAL AND METHODS

Sampling

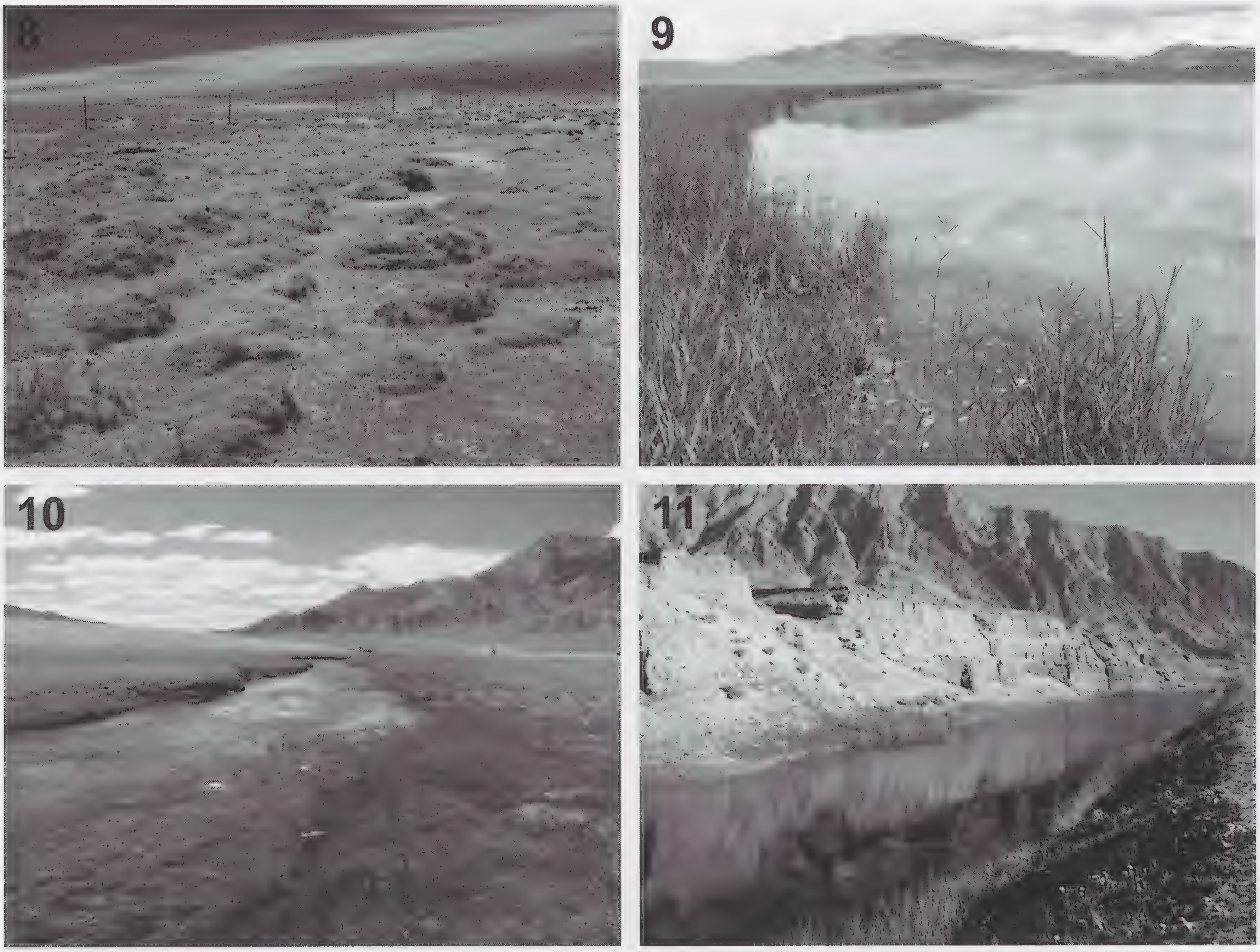
During field work conducted between 2009 and 2012, specimens of the family Sphaeriidae were found at 27 locations throughout the Tibetan Plateau (Fig. 7). Specimens were collected by sieving bottom substrate of several types of freshwater habitats, mostly lakes, rivers, ponds, and wetlands (Figs. 8–11) and preserved in 80% ethanol. For comparison, additional sphaeriid specimens were included from 39 localities in parts of Asia (especially locations in the south of the Himalayas, six species from Nepal), Europe and Africa (Appendix 1).

Species determination was performed based on shell morphology (soft tissue removed by boiling in 3% sodium hydroxide solution) and soft body anatomy. Vouchers (DNA, shells) are stored at the Systematics and Biodiversity collection of the University of Giessen (UGSB; Appendix 1). For morphologically determined sphaeriids from some Tibetan Plateau locations, no molecular data could be obtained (Appendix 1).

Given the conflicting taxonomic concepts among Sphaeriidae (Graf, 2013), we here use the traditional nomenclature on species level (according to Korniushin & Glaubrecht, 2002), while classification of the clades inferred is based on the phylogenetic analyses of Lee & Ó Foighil (2003). Acknowledging that the purpose of this paper is not primarily taxonomic, future studies based on more comprehensive global datasets should address the internal classification of Sphaeriidae.

DNA Isolation, PCR, and Sequencing

This study includes new genetic data for a total of 101 specimens, including four specimens of *Eupera* spp. as outgroup (Appendix 1). Genomic DNA was extracted from individual



FIGS. 8–11. Representatives of the four dominant habitat types inhabited by sphaeriid species on the Tibetan Plateau. FIG. 8: Wetland (location CN05); FIG. 9: Lake (CN21); FIG. 10: River (CN26); FIG. 11: Pond (CN01).

clams using the CTAB protocol described by Wilke et al. (2006). For phylogenetic analyses, we obtained sequences from fragments of three genes: (1) mitochondrial cytochrome oxidase *c* subunit I (COI), (2) mitochondrial large ribosomal subunit (mtLSU rRNA or 16S) and (3) nuclear large ribosomal subunit (nLSU rRNA or 28S). Standard and modified primers were used for the amplification of COI: LCO1490 (Folmer et al., 1994) or a modified version (lacking 5 bases from the 5' end) and a modified version of HCO2198 (Folmer et al., 1994; minus the first 6 bases at the 5' end). The latter has already been used before especially for freshwater bivalves (Hoeh et al., 2002). Forward and reverse primers for 16S were taken from Palumbi et al. (1991; 16Sar-L and 16Sbr-H) and for 28S from Park & Ó Foighil (2000; D23F and D6R).

PCR cycling conditions were as follows: an initial denaturation step at 95°C for 60 s, followed by 30–36 amplification cycles (denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 30 s), depending on the concentration of genomic DNA. The reaction was terminated after a final extension step at 72°C for 180 s. Sequences (forward and reverse) were visualized on an ABI 3730 XL sequencer (Life Technologies) using a Big Dye Terminator Kit (Life Technologies).

In addition, 16S sequences from 49 sphaeriid specimens were obtained from GenBank (Appendix 2).

Sequence Alignment

The alignments of sequences were done individually for all three fragments (68 COI, 146 16S, and 32 28S). The first base pairs (bp) behind the 3' end of each primer were difficult to read in all fragments. Therefore, we trimmed each sequence, leaving a 599 bp-long COI fragment, a 741–743 bp-long 28S fragment, and a 472–503 bp-long 16S fragment. The COI and 28S sequences could be unambiguously aligned using ClustalW implemented in BioEdit 7.0.8.0 (Hall, 1999). The alignment of 16S sequences was performed following the instructions for structural alignment by Kjer et al. (2009) based on the LSU rRNA structure model of *Mytilus edulis* (Hoffmann et al., 1992). In total, three regions were excluded from the 16S dataset because no reliable alignment could be achieved (positions 66–80, 281–296, and 341–354), leaving a final alignment of 471 bp (the aligned dataset is available from the corresponding author upon request).

Phylogenetic Analyses

The subsequent analyses (except network analyses) were performed using all 119 unique haplotypes of a combined dataset of COI, 16S, and 28S sequences.

The COI, 16S, and 28S partitions were separately tested for substitution saturation using DAMBE 5.2.73 (Xia et al., 2003; Xia & Lemey, 2009). Under the assumption of a symmetrical as well as an asymmetrical tree, the test showed only little saturation for all three partitions.

Phylogenetic relationships were reconstructed using Bayesian inference implemented in the software package MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001; parameters: nchains = 4, ngen = 8,000,000, samplefreq = 20, temp = 0.1). Prior to the analyses, jModelTest 0.1.1 (Posada, 2008) was used to find the best fit model of sequence evolution for the individual datasets based on the corrected Akaike Information Criterion (AICc). For the COI partition the program selected the HKY+I+G model and for the 28S partition the TIM3+I+G model. Two different models were applied for 16S based on the structural alignment: the 16B model (Schöniger & Haeseler, 1994) for the stem regions and the HKY+G model for the loop regions.

Monitored in Tracer v1.5.0 (Drummond & Rambaut, 2007), the combined set of trees showed both high ESS (effective sample size) values (> 300 for all parameters) and a smooth frequency plot. For the consensus tree the first 25% of the sampled trees (N = 100,000) were discarded as burn-in.

Phylogeographical Analyses

We performed a parsimony network analysis for the most widespread and most abundant species *P. stewarti* using TCS 1.21 (Clement et al., 2000) and a combined dataset of COI and 16S sequences. The resulting dataset contained 14 specimens from nine locations across the Tibetan Plateau.

RESULTS

Diversity, Distribution and Ecology

A total of six sphaeriid species were sampled across the 27 localities on the Tibetan Plateau including five species of *Pisidium* – *P. stewarti* Preston, 1909; *P. subtruncatum* Malm, 1855;

P. zugmayeri Weber, 1910; *P. nitidum* Jenyns, 1832; *Pisidium* sp. I – and one species of *Musculium, M. kashmirensis* (Prashad, 1937) (Figs. 1–6). *Pisidium* sp. I represents an undetermined but morphologically distinct species from the clade *Cyclocalyx*. The Tibetan Plateau taxa occurred in different habitats (Table 1). Only specimens of *P. stewarti* and *M. kashmirensis* inhabited all major habitat types: wetlands, lakes, rivers and ponds. Lakes were the most species-rich habitat type (five species). The most frequent species on the Tibetan Plateau was *P. stewarti*, found at 21 out of 27 locations (77.8%). With a frequency of 29.6% (found at eight locations), *M. kashmirensis* was the second most common species. The remaining species were relatively rare: *P. nitidum* and *P. zugmayeri* were each observed at four different locations (14.8%); *P. subtruncatum* and *Pisidium* sp. I were collected at three locations (11.5%). At 12 sampling sites (Table 1) two or more sphaeriid species occurred in sympatry (up to four at the same location). A total of seven different species assemblages were found. The most frequent composition was *M. kashmirensis* together with *P. stewarti* (in 5 out of 12 locations; Fig. 7).

General distribution data of Tibetan Plateau sphaeriids were retrieved from the literature and the present sampling (Table 2). *Pisidium nitidum* and *P. subtruncatum* are both known to have a large distribution range inhabiting several parts of the Holarctic; *P. zugmayeri* is probably endemic to the Tibetan Plateau. The Recent distribution of *P. stewarti* is restricted to areas with cold climates in central Asia and Siberia. *Musculium kashmirensis* is distributed in Kashmir, Nepal and on the Tibetan Plateau. Note that another species, *M. goshaitanensis* Nesemann & Sharman, 2005, was described from Punyamata, Kathmandu, Bagmati, Nepal.

Phylogenetic Analyses

The Bayesian analysis revealed a tree with five major clades (Fig. 12), which correspond to the major lineages of Lee & Ó Foighil (2003): *Sphaerium*, *Pisidium* s. str., *Cyclocalyx*, *Odhneripisidium* and *Afropisidium*. The topology is unresolved for basal as well as for few terminal nodes. Four of the major clades are well supported with a Bayesian Posterior Probability (BPP) ≥ 0.95. The genus *Pisidium* appears paraphyletic in the phylogeny as the clade comprising *Pisidium* s. str., including specimens of *P. amnicum* (O. F. Müller, 1774) and *P. dubium* (Say, 1817), forms the sister clade of the *Sphaerium* clade, whereas *Cyclocalyx* is the sister group to the former two clades.

The six sphaeriid species of the Tibetan Plateau do not form a monophyletic group and belong to the clades of *Sphaerium*, *Cyclocalyx* and *Odhneripisidium*. The clades of *Afropisidium* and *Pisidium* s. str. are not present on the Tibetan Plateau. The phylogenetic relationships of the Tibetan Plateau sphaeriids to their respective sister groups are described as following (Fig. 12):

(I) The Tibetan Plateau *M. kashmirensis* are not monophyletic but form a well-supported but unresolved clade (BPP

TABLE 1. Sphaeriid species found among sampling sites on the Tibetan Plateau. N_S = number of species per habitat type; N_L = number of locations where the respective species was found; F = frequency of species; Habitat Types: wetlands, lakes, rivers, ponds (‘others’ are described in detail in Appendix 1); CN01–CN27 = location codes (see also Fig. 7), * = sympatry occurring.

	Wetlands							Lakes						Rivers					Ponds					others					
Species	CN03*	CN04	CN05	CN11	CN12	CN18*	CN22	CN07*	CN13	CN10*	CN15	CN17	CN21*	CN02	CN16*	CN19*	CN26*	CN27	CN01	CN06	CN08*	CN09	CN23	CN14*	CN20*	CN24*	CN25	N_L	F (%)
M. kashmirensis	x		x							x					x	x		x			x						x	8	29.6
P. nitidum								x					x												x	x		4	14.8
P. stewarti	x	x		x	x	x		x	x	x	x	x		x	x	x	x		x	x	x	x	x		x	x		21	77.8
P. subtruncatum													x											x		x		3	11.1
P. zugmayeri						x	x										x									x		4	14.8
Pisidium sp. I								x					x											x				3	11.1
N_S				3						5						3					2				6				

TABLE 2. Species of the family Sphaeriidae collected on the Tibetan Plateau with recent distribution and range type (except for *Pisidium* sp. I) based on the literature and own data.

Species		Recent distribution	Range type
M. kashmirensis	(Prashad, 1937)	Tibetan Plateau[1], Kashmir[2], Nepal[1]	restricted
P. nitidum	Jenyns, 1832	Holarctic[3]	widespread
P. stewarti	Preston, 1909	central Asia, north of the Himalayas[4,5,6]	restricted (to cold climates)
P. subtruncatum	Malm, 1855	Holarctic[3]	widespread
P. zugmayeri	Weber, 1910	Tibetan Plateau[7,8]	endemic

[1]this study; [2]Prashad, 1937; [3]Kuiper et al., 1989; [4]Preston, 1909; [5]Bogan, 2011; [6]Bössneck, unpubl. data; [7]Prashad, 1933; [8]Mischke et al., 2010

1.00) with *M. kashmirensis* from Nepal, *M. japonicum* (Westerlund, 1883) from Japan and *M. miyadii* Mori, 1993, from Russia (Kuril Islands). The relationship of this clade to *M. lacustre* (O. F. Müller, 1774) from Germany, Georgia and the U.S.A., is likewise unresolved (BPP 0.96).

(II) *Pisidium* sp. I (clade *Cyclocalyx*) is not monophyletic but clusters with *P. casertanum* (Poli, 1791) from Germany (BPP 0.68). The sister of this clade is a specimen of *P. atkinsonianum* Theobald, 1876 from Nepal (BPP 0.62). Note also that *P. casertanum* is not monophyletic according to the phylogeny of the present study.

(III) The Tibetan Plateau specimens of *P. nitidum* (clade *Cyclocalyx*) form no monophyletic group but cluster together with other *P. nitidum* specimens and specimens of three other species (*P. edlaueri* Kuiper, 1960; *P. milium* Held, 1836; and *P. pseudosphaerium* J. Favre, 1927) from Germany, Macedonia and Turkey in an unresolved clade (BPP 0.52).

(IV) The Tibetan Plateau *P. subtruncatum* (clade *Cyclocalyx*) are monophyletic (BPP 1.00) and sister to other specimens of this species from Georgia, Italy and Macedonia, together forming a well supported clade (BPP 0.99).

(V) *Pisidium zugmayeri* (clade *Cyclocalyx*) forms a monophyletic group (BPP 1.00) that falls into a strongly supported clade of *P. casertanum* from Ireland, Macedonia and Turkey (BPP 0.99); thereby, the specimen from Ireland is the direct sister to the Tibetan Plateau specimens (BPP 1.00).

(VI) The specimens of *P. stewarti* represent a distinct monophyletic group (BPP 1.00) within the *Odhneripisidium* clade, being sister to *P. moitesserianum* (Paladilhe, 1866) (BPP 0.71).

The genetic distance between *P. stewarti* and its sister group; however, appears to be high as revealed by long branches from their common ancestor. For *P. stewarti* a haplotype network analysis was conducted (Fig. 13).

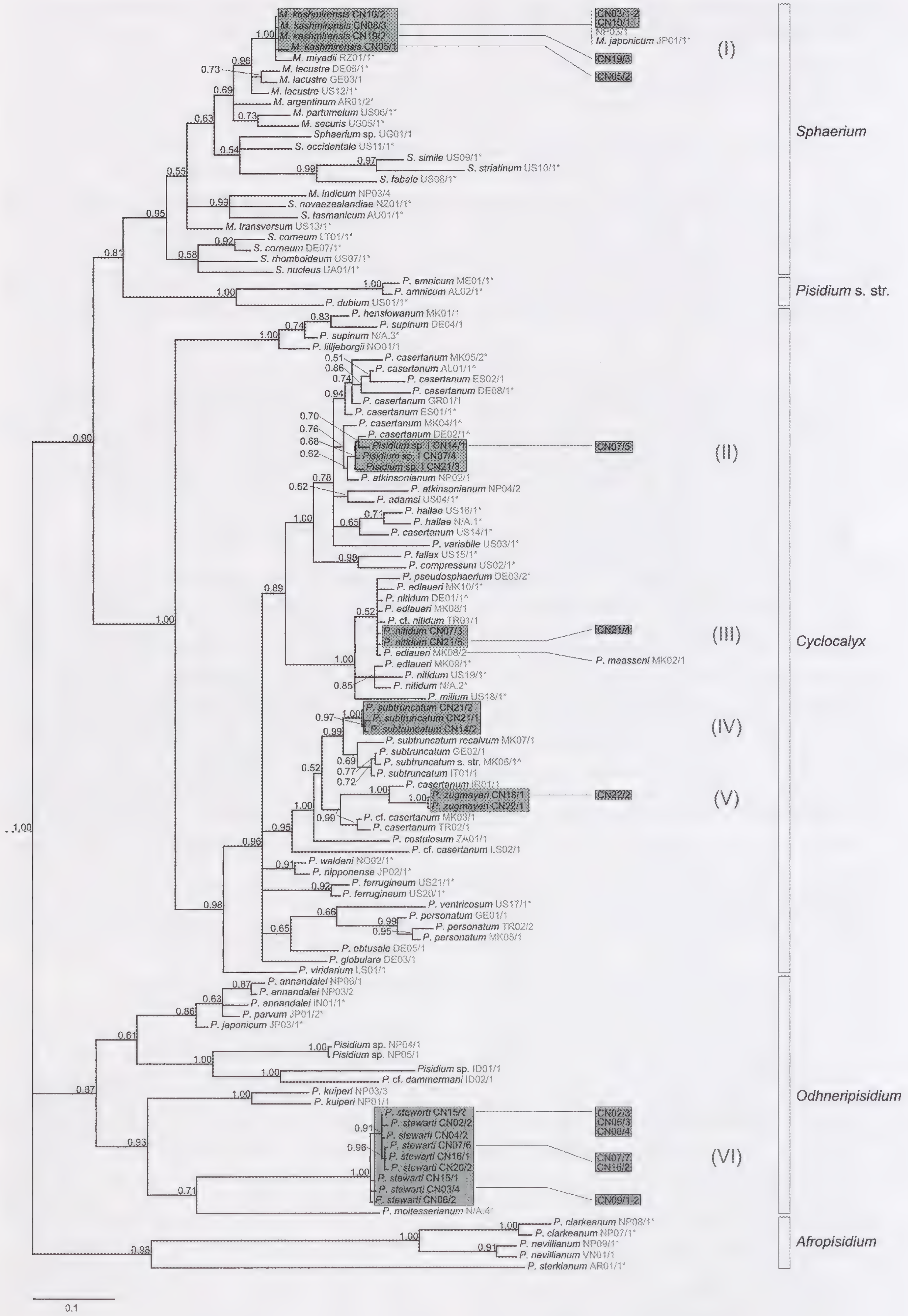
Phylogeographical Analyses

The parsimony network analysis of 14 *P. stewarti* specimens from the Tibetan Plateau revealed nine unique haplotypes for the combined dataset of 16S and COI. These haplotypes fall into two subclades (A: 6 haplotypes, B: 3 haplotypes) separated by eight mutational steps (Fig. 13). Specimens from four geographically very distant locations (max. ca. 1,400 km) share the most frequent haplotype (subclade A in Fig. 13). There is no obvious correlation between genetic distances (mutational steps) and geographical distances on the plateau (map in Fig. 13).

DISCUSSION

Diversity, Distribution and Ecology

Prior to the present study, little was known about sphaeriid distribution in freshwater systems throughout the Tibetan Plateau. Besides the records of *P. zugmayeri* in lakes Bangong Co (western Tibet; Prashad, 1933) and Donggi Cona (eastern Tibet; Mischke et al., 2010), there were only few other records of *P. stewarti* from the plateau (Preston, 1909; Dance, 1967). This study, however, demonstrates that the diversity of the Sphaeriidae from the Tibetan Plateau is significantly higher than previously known, as revealed by the first records of *M. kashmirensis*, *P. subtruncatum*, *P. nitidum* and *Pisidium* sp. I. Additionally, a recent drilling core analysis by Mischke et al. (unpubl. data;


M. kashmirensis CN10/2
M. kashmirensis CN08/3
M. kashmirensis CN19/2
M. kashmirensis CN05/1
M. miyadii RZ01/1*
M. lacustre DE06/1*
M. lacustre GE03/1
M. lacustre US12/1*
M. argentinum AR01/2*
M. partumeium US06/1*
M. securis US05/1*
Sphaerium sp. UG01/1
S. occidentale US11/1*
S. simile US09/1*
S. striatinum US10/1*
S. fabale US08/1*
M. indicum NP03/4
S. novaezealandiae NZ01/1*
S. tasmanicum AU01/1*
M. transversum US13/1*
S. corneum LT01/1*
S. corneum DE07/1*
S. rhomboideum US07/1*
S. nucleus UA01/1*
P. amnicum ME01/1*
P. amnicum AL02/1*
P. dubium US01/1*
P. henslowanum MK01/1
P. supinum DE04/1
P. supinum N/A.3*
P. lilljeborgii NO01/1
P. casertanum MK05/2*
P. casertanum AL01/1^
P. casertanum ES02/1
P. casertanum DE08/1*
P. casertanum GR01/1
P. casertanum ES01/1*
P. casertanum MK04/1^
P. casertanum DE02/1^
Pisidium sp. I CN14/1
Pisidium sp. I CN07/4
Pisidium sp. I CN21/3
P. atkinsonianum NP02/1
P. atkinsonianum NP04/2
P. adamsi US04/1*
P. hallae US16/1*
P. hallae N/A.1*
P. casertanum US14/1*
P. variabile US03/1*
P. fallax US15/1*
P. compressum US02/1*
P. pseudosphaerium DE03/2*
P. edlaueri MK10/1*
P. nitidum DE01/1^
P. edlaueri MK08/1
P. cf. nitidum TR01/1
P. nitidum CN07/3
P. nitidum CN21/5
P. edlaueri MK08/2
P. edlaueri MK09/1*
P. nitidum US19/1*
P. nitidum N/A.2*
P. milium US18/1*
P. subtruncatum CN21/2
P. subtruncatum CN21/1
P. subtruncatum CN14/2
P. subtruncatum recalvum MK07/1
P. subtruncatum GE02/1
P. subtruncatum s. str. MK06/1^
P. subtruncatum IT01/1
P. casertanum IR01/1
P. zugmayeri CN18/1
P. zugmayeri CN22/1
P. cf. casertanum MK03/1
P. casertanum TR02/1
P. costulosum ZA01/1
P. cf. casertanum LS02/1
P. waldeni NO02/1*
P. nipponense JP02/1*
P. ferrugineum US21/1*
P. ferrugineum US20/1*
P. ventricosum US17/1*
P. personatum GE01/1
P. personatum TR02/2
P. personatum MK05/1
P. obtusale DE05/1
P. globulare DE03/1
P. viridarium LS01/1
P. annandalei NP06/1
P. annandalei NP03/2
P. annandalei IN01/1*
P. parvum JP01/2*
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P. cf. dammermani ID02/1
P. kuiperi NP03/3
P. kuiperi NP01/1
P. stewarti CN15/2
P. stewarti CN02/2
P. stewarti CN04/2
P. stewarti CN07/6
P. stewarti CN16/1
P. stewarti CN20/2
P. stewarti CN15/1
P. stewarti CN03/4
P. stewarti CN06/2
P. moitesserianum N/A.4*
P. clarkeanum NP08/1*
P. clarkeanum NP07/1*
P. nevillianum NP09/1*
P. nevillianum VN01/1
P. sterkianum AR01/1*
CN03/1-2
CN10/1
NP03/1
M. japonicum JP01/1*
CN19/3
CN05/2
CN07/5
CN21/4
P. maasseni MK02/1
CN22/2
CN02/3
CN06/3
CN08/4
CN07/7
CN16/2
CN09/1-2
(I)
(II)
(III)
(IV)
(V)
(VI)
Sphaerium
Pisidium s. str.
Cyclocalyx
Odhneripisidium
Afropisidium
1.00
0.96
0.73
0.69
0.73
0.63
0.54
0.97
0.99
0.55
0.99
0.95
0.92
0.81
0.58
1.00
1.00
0.83
0.74
1.00
0.51
0.86
0.74
0.94
0.70
0.76
0.68
0.62
0.78
0.62
0.71
0.65
1.00
0.98
0.90
0.89
0.52
1.00
1.00
0.85
1.00
0.97
0.99
0.69
0.77
0.72
0.52
1.00
1.00
1.00
0.99
0.95
0.96
0.91
0.92
0.66
0.99
0.95
0.65
0.98
1.00
0.87
0.63
0.86
0.61
1.00
1.00
1.00
0.87
1.00
0.91
0.96
0.93
1.00
0.71
1.00
1.00
0.91
0.98
0.1

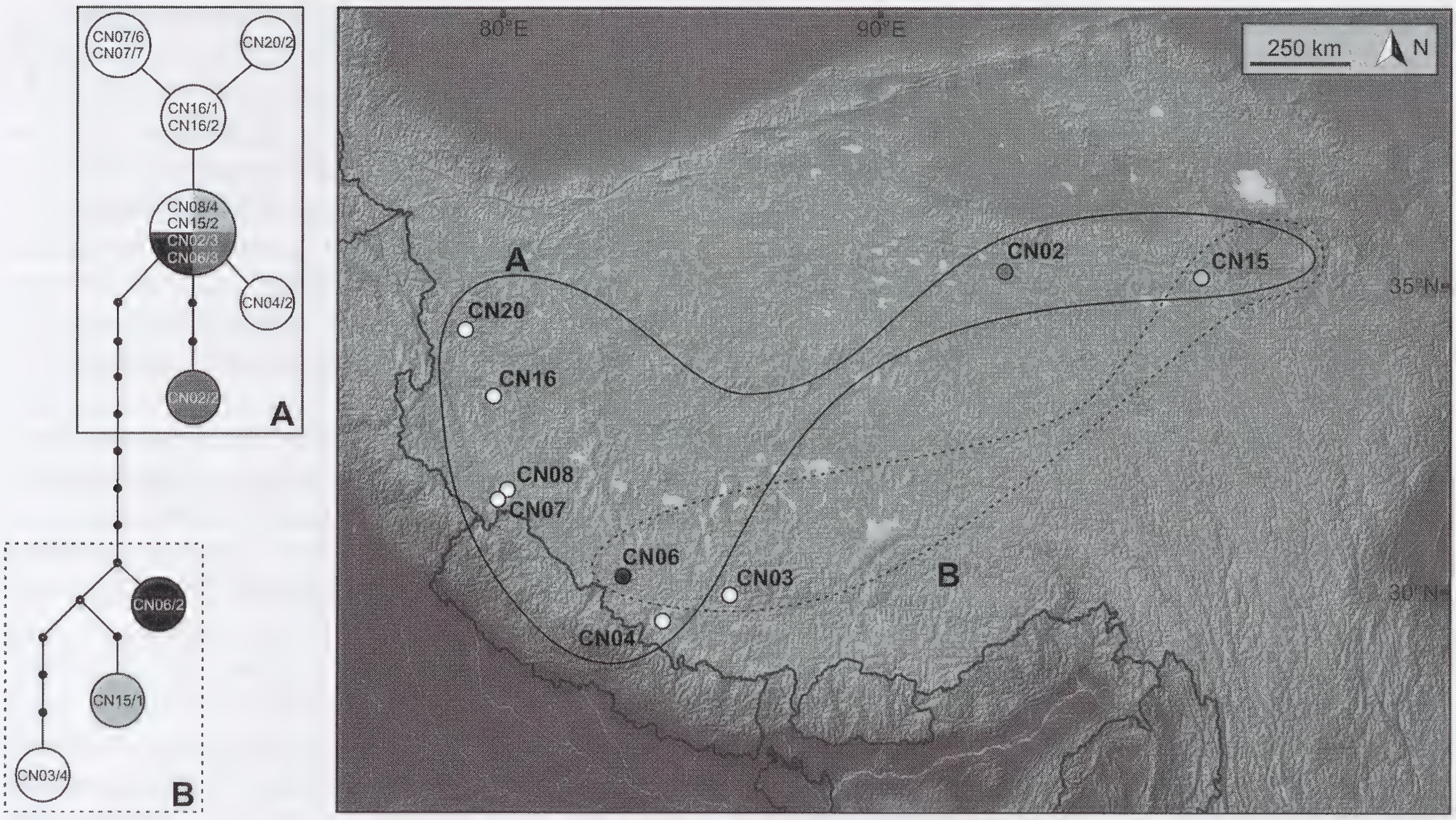

FIG. 13. Statistical parsimony network of *P. stewarti* from the Tibetan Plateau. The geographic distribution of subgroups (A, B) is shown in the map. Locations including more than one haplotype are labeled in different grayscales.

ca. 16,000 years ago; Luanhaizi Lake, Qinghai province, northeastern Tibetan Plateau, China) has revealed shell fragments of *P. obtusale* (Lamarck, 1818). Hence, additional sampling might reveal even more sphaeriid species on the Tibetan Plateau.

Sphaeriids are able to occupy a great variety of habitats, however, the highest species diversity is observed in freshwater lakes, ponds, and small rivers (Korniushin, 2006). Some sphaeriid species show clear preferences to particular habitats (e.g., *P. lilljeborgi* Clessin, 1886: lakes; *P. supinum* Schmidt, 1851: rivers), others are generalists inhabiting a wide range of habitats. Thus, such generalists seem to be less sensitive to changes in environmental conditions (e.g., oxygen content; Korniushin, 2006). This fact might be of advantage for generalist species occurring in extreme habitats (i.e., high mountain ranges like the Tibetan Plateau) with partial seasonal freezing, increased ultraviolet radiation and low oxygen level. Interestingly, *P. stewarti* and *M. kashmirensis* were present in all major habitat types; the remaining species appear to be more restricted to particular habitats (Table 1). This habitat occupation pattern might be due to an increased specialization of species at the high elevations of the Tibetan Plateau. For instance, *P. nitidum* and *P. subtruncatum* generally inhabit lotic (flowing) as well as lentic (stagnant) waters (Kuiper et al., 1989), but they have been only

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FIG. 12. Bayesian phylogram of Sphaeriidae (comprising the genera *Musculium*, *Sphaerium*, and *Pisidium*) based on COI, mtLSU rRNA (16S), and nLSU rRNS (28S) sequences. Bayesian posterior probabilities (higher than 0.5) are given next to the respective node. Individuals from the Tibetan Plateau are highlighted in gray and consecutively numbered with roman numerals (see also Fig. 1). The scale bar represents substitutions per site according to the applied model of sequence evolution. Major clades are marked with white bars according to the classification of Lee & Ó Foighil (2003). Specimens (sequences) exclusively from GenBank are marked with asterisks (*), such with additional new COI sequence data are marked with circumflex accents (^). Outgroups (*Eupera* spp.), with the specimen codes: MW01/1, NA01/1, and UG01/1–2, are not shown. Specimens sharing a haplotype are provided next to the phylogram except for CN01/1, CN02/1, CN03/3, CN04/1, CN06/1, CN07/1–2, CN08/1–2, CN11/1, CN12/1, CN13/1, CN17/1, CN19/1, and CN20/1, which share the same 16S haplotype with CN15/2 and of which no COI data are available. For detailed specimen information see Appendices 1 and 2.

recorded from lakes on the plateau. However, a sampling artifact cannot be ruled out due to the small sample sizes (N = max. 7) for each habitat type. Nonetheless, the highest species diversity was observed for the habitat type "lakes" (see above), which may be caused by a higher diversity of microhabitats in lakes, or by the larger size and higher stability of lakes compared to ponds and wetlands.

Recent studies on Tibetan Plateau freshwater gastropods have detected three to four distinct genetic lineages for *Radix* (Oheimb et al., 2011), two lineages for *Gyraulus* (Oheimb et al., 2013) and only one lineage for *Valvata* (unpubl. data). Thus, the diversity of Tibetan Plateau sphaeriids seems to be slightly higher. A relatively high species diversity of sphaeriids is also reported for other high mountain ranges such as the Andes in South America, as well as Mt. Kilimanjaro and Mt. Kenya in eastern Africa (Bössneck, 2012). General observations of the freshwater mollusk fauna in high mountainous regions show that the gastropod-dominated fauna at lower elevations is replaced by a sphaeriid-dominated fauna with increasing elevation (Bössneck, 2012). In conclusion, the Sphaeriidae appear to be one of the few freshwater mollusk taxa being well adapted to high mountainous regions.

Biogeography of Tibetan Plateau Sphaeriids

Given that the Tibetan Plateau sphaeriids do not constitute a monophyletic group (Fig. 12), independent colonizations of the plateau have to be assumed. We found at least six independent colonization events; four within the clade *Cyclocalyx,* one for *Odhneripisidium* and one for the clade *Sphaerium*, each representing a single species lineage. Phylogenetic analyses of the Tibetan Plateau freshwater gastropod genera *Radix* and *Gyraulus* revealed at least three and two independent colonization events of the plateau by these taxa, respectively (Oheimb et al., 2011, 2013). Apparently, there are decisive differences between these three mollusk taxa resulting in a potentially higher colonization rate of sphaeriids. One major difference possibly causing the increased colonization rate is the high dispersal capacity in sphaeriids (see Introduction).

In order to infer the biogeographical relationships of the Tibetan Plateau sphaeriid species, knowledge about the zoogeographical affinities of the respective sister groups are useful. Generally, the sister groups are dominated by species from northern Eurasia (Palearctic region). Based on the general distribution of the respective species (Table 2) and the close relationship to Palearctic taxa, *P. nitidum*, *P. subtruncatum* and *P. zugmayeri* can be considered as Palearctic elements of the Tibetan Plateau fauna. The affinity of *Pisidium* sp. I, however, remains questionable due to the weakly supported relationships to their potential sister taxa, the Palearctic species *P. casertanum* and the species *P. atkinsonianum* (Fig. 12).

In the case of *M. kashmirensis* several specimens from the Tibetan Plateau as well as from Far East Russia and Japan (Palearctic region) cluster with one specimen from the Oriental region of Nepal. In contrast to *Gyraulus* spp. (Oheimb et al., 2013), *M. kashmirensis* may thus have crossed the Himalayan mountain range. However, for *M. kashmirensis* from Nepal we could only generate sequence data from the relatively conservative 16S gene but not from COI. Furthermore, this species has been collected in Nepal only in the highly frequented Kathmandu valley and thus may have been recently introduced there. As the holotype of *M. kashmirensis* was described from Kashmir (Prashad, 1937), a region with both Palearctic and Oriental elements (Das, 1966), the zoogeographical affinity of Tibetan Plateau *M. kashmirensis* remains ambiguous.

The sister species of *P. stewarti* is *P. moitesserianum* in our phylogeny, however, the relationship is characterized by relatively long branches. Although locality information for the respective *P. moitesserianum* specimen was not provided with the original GenBank data, the distribution of this species is known to be restricted to Eurasia (Zettler & Kuiper, 2002), and it thus most likely has a Palearctic origin.

Additionally, Pleistocene fossils of *P. stewarti* described from several parts in Europe (Briggs & Gilbertson, 1980; Ložek, 2001) also suggest Palearctic affinities for this species.

In contrast to other Tibetan Plateau sphaeriids (i.e., *P. subtruncatum* and *P. nitidum* with a Holarctic distribution), *P. stewarti* often occurs on high elevations in central Asia, with a remarkably wide range across the plateau. Therefore, this species was used for a more detailed haplotype network analysis (Fig. 13). The results of this analysis suggest high dispersal rates throughout the Tibetan Plateau. This is illustrated by specimens from geographically very distant locations (i.e., ca. 1,400 km) which share the same haplotype. In contrast, the maximum geographical distance between locations where identical haplotypes have been found is approx. 450 km and 300 km for *Gyraulus* and *Radix*, respectively (Oheimb et al., 2011, 2013). This observation highlights the high efficiency of sphaeriid dispersal. Nevertheless, the Himalayan mountain range also represents a crucial biogeographical barrier for sphaeriids, as for other Tibetan Plateau freshwater mollusks. This might be exemplified by Palearctic affinities of most of the Tibetan Plateau sphaeriids. Furthermore, this fauna is conspicuously different from the adjacent Oriental Sphaeriidae fauna from Nepal comprising ten different and only one shared species (*M. kashmirensis*) (see also Korniushin & Bössneck, 2003; Nesemann & Sharma, 2005).

CONCLUSION

The present study demonstrates that the Tibetan Plateau Sphaeriidae fauna is characterized by a previously unknown high species diversity, which is dominated by Palearctic elements. A total of six different species have been recorded, four of them for the first time on the Tibetan Plateau. Furthermore, our study supports the hypothesis that the Himalayan mountain range represents a crucial biogeographical barrier for freshwater mollusks.

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APPENDIX 1. List of studied specimens including specimen code (^ = specimens with 16S from GenBank and COI sequences produced by ourselves), taxon, locality information, voucher details (materials are stored at the Systematics and Biodiversity Collection of the University of Giessen – UGSB), and GenBank accession numbers.

Spec. Code	Taxon	Locality	Latitude	Longitude	Spec. voucher #	COI GenBank acc.#	16S GenBank acc.#	28S GenBank acc.#
AL01/1^	*Pisidium casertanum*	Albania, Qark Korça	40.89623°	20.71040°	UGSB 10544	KF483356	EU559094	
CN01/1	*Pisidium stewarti*	China, Tibet, pond south of Golmud	35.88329°	94.57995°	UGSB 8391		KF483234	
CN02/1	*Pisidium stewarti*	China, Tibet, small river south of Golmud	35.43374°	93.60115°	UGSB 8392		KF483235	
CN02/2	*Pisidium stewarti*	China, Tibet, small river south of Golmud	35.43374°	93.60115°	UGSB 9988	KF483357	KF483236	
CN02/3	*Pisidium stewarti*	China, Tibet, small river south of Golmud	35.43374°	93.60115°	UGSB 9990	KF483358	KF483237	
CN03/1	*Musculium kashmirensis*	China, Tibet, wetlands west of Ngamring Co	29.34203°	87.16489°	UGSB 8394		KF483238	
CN03/2	*Musculium kashmirensis*	China, Tibet, wetlands west of Ngamring Co	29.34203°	87.16489°	UGSB 8395	KF483359	KF483239	
CN03/3	*Pisidium stewarti*	China, Tibet, wetlands west of Ngamring Co	29.34203°	87.16489°	UGSB 8396		KF483240	
CN03/4	*Pisidium stewarti*	China, Tibet, wetlands west of Ngamring Co	29.34203°	87.16489°	UGSB 9992	KF483360	KF483241	
CN04/1	*Pisidium stewarti*	China, Tibet, wetlands near Paiku Co	28.77180°	85.56133°	UGSB 8398		KF483242	
CN04/2	*Pisidium stewarti*	China, Tibet, wetlands near Paiku Co	28.77180°	85.56133°	UGSB 9995	KF483361	KF483243	
CN05/1	*Musculium kashmirensis*	China, Tibet, wetlands west of Saga	29.40823°	85.24297°	UGSB 8400		KF483245	

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Spec. Code	Taxon	Locality	Latitude	Longitude	Spec. voucher #	COI Gen-Bank acc.#	16S Gen-Bank acc.#	28S Gen-Bank acc.#
CN05/2	*Musculium kashmirensis*	China, Tibet, wetlands west of Saga	29.40823°	85.24297°	UGSB 8401		KF483246	
CN06/1	*Pisidium stewarti*	China, Tibet, pond near Lazangxiang	29.50262°	84.57673°	UGSB 8403		KF483244	
CN06/2	*Pisidium stewarti*	China, Tibet, pond near Lazangxiang	29.50262°	84.57673°	UGSB 9997	KF483362	KF483247	
CN06/3	*Pisidium stewarti*	China, Tibet, pond near Lazangxiang	29.50262°	84.57673°	UGSB 9998	KF483363	KF483248	
CN07/1	*Pisidium stewarti*	China, Tibet, Lake Manasarovar	30.60952°	81.36448°	UGSB 8404		KF483249	
CN07/2	*Pisidium stewarti*	China, Tibet, Lake Manasarovar	30.60952°	81.36448°	UGSB 8405		KF483250	
CN07/3	*Pisidium nitidum*	China, Tibet, Lake Manasarovar	30.60952°	81.36448°	UGSB 8406	KF483364	KF483251	
CN07/4	*Pisidium* sp. I	China, Tibet, Lake Manasarovar	30.60952°	81.36448°	UGSB 8407	KF483365	KF483252	KF483324
CN07/5	*Pisidium* sp. I	China, Tibet, Lake Manasarovar	30.60952°	81.36448°	UGSB 8408		KF483253	
CN07/6	*Pisidium stewarti*	China, Tibet, Lake Manasarovar	30.60952°	81.36448°	UGSB 10001	KF483366	KF483254	
CN07/7	*Pisidium stewarti*	China, Tibet, Lake Manasarovar	30.60952°	81.36448°	UGSB 10002	KF483367	KF483255	
CN08/1	*Pisidium stewarti*	China, Tibet, spring complex north of Lake Manasarovar	30.81483°	81.56236°	UGSB 8409		KF483256	
CN08/2	*Pisidium stewarti*	China, Tibet, spring complex north of Lake Manasarovar	30.81483°	81.56236°	UGSB 8410		KF483257	

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Spec. Code	Taxon	Locality	Latitude	Longitude	Spec. voucher #	COI Gen-Bank acc.#	16S Gen-Bank acc.#	28S Gen-Bank acc.#
CN08/3	*Musculium kashmirensis*	China, Tibet, spring complex north of Lake Manasarovar	30.81483°	81.56236°	UGSB 8412	KF483368	KF483258	
CN08/4	*Pisidium stewarti*	China, Tibet, spring complex north of Lake Manasarovar	30.81483°	81.56236°	UGSB 10003	KF483369	KF483259	
CN09/1	*Pisidium stewarti*	China, Tibet, pond near Cam Co	32.08300°	83.66180°	UGSB 8376		KF483260	
CN09/2	*Pisidium stewarti*	China, Tibet, pond near Cam Co	32.08300°	83.66180°	UGSB 8377		KF483261	
CN10/1	*Musculium kashmirensis*	China, Tibet, Garing Co	30.76610°	85.01230°	UGSB 8378		KF483262	
CN10/2	*Musculium kashmirensis*	China, Tibet, Garing Co	30.76610°	85.01230°	UGSB 8379	KF483370	KF483263	KF483325
CN10/3	*Pisidium stewarti*	China, Tibet, Garing Co	30.76610°	85.01230°	UGSB 12570		no sequence information	
CN11/1	*Pisidium stewarti*	China, Tibet, wetlands south of Gerze	32.06443°	83.90559°	UGSB 8383		KF483264	
CN12/1	*Pisidium stewarti*	China, Tibet, wetlands west of Cakaxiang	32.44139°	82.43805°	UGSB 8384		KF483265	
CN13/1	*Pisidium stewarti*	China, Tibet, Dajiamang Co	29.63267°	85.74019°	UGSB 8388		KF483266	
CN14/1	*Pisidium* sp. I	China, Qinghai, artificial dam region near Donggi Cona	35.35419°	98.34894°	UGSB 9087	KF483371	KF483269	KF483326
CN14/2	*Pisidium subtruncatum*	China, Qinghai, artificial dam region near Donggi Cona	35.35419°	98.34894°	UGSB 9088	KF483372	KF483270	KF483327
CN15/1	*Pisidium stewarti*	China, Qinghai, Donggi Cona	35.28861°	98.70116°	UGSB 9089	KF483373	KF483267	KF483328
CN15/2	*Pisidium stewarti*	China, Qinghai, Donggi Cona	35.28861°	98.70116°	UGSB 9090	KF483374	KF483268	KF483329
CN16/1	*Pisidium stewarti*	China, Tibet, river west of Ge’gyai	32.45972°	80.89935°	UGSB 10006	KF483375	KF483271	
CN16/2	*Pisidium stewarti*	China, Tibet, river west of Ge’gyai	32.45972°	80.89935°	UGSB 10007	KF483376	KF483272	

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Spec. Code	Taxon	Locality	Latitude	Longitude	Spec. voucher #	COI Gen-Bank acc.#	16S Gen-Bank acc.#	28S Gen-Bank acc.#
CN16/3	*Musculium kashmirensis*	China, Tibet, river west of Ge'gyai	32.45972°	80.89935°	UGSB 12571		no sequence information	
CN17/1	*Pisidium stewarti*	China, Tibet, river near Nam Co	30.47651°	90.20139°	UGSB 10987		KF483273	KF483355
CN18/1	*Pisidium zugmayeri*	China, Tibet, wetlands north of Bangong Co	33.62848°	79.76558°	UGSB 10989	KF483377	KF483275	
CN18/2	*Pisidium stewarti*	China, Tibet, wetlands north of Bangong Co	33.62848°	79.76558°	UGSB 10461		no sequence information	
CN19/1	*Pisidium stewarti*	China, Tibet, inflow Bangong Co	33.66933°	80.46586°	UGSB 10990		KF483274	KF483352
CN19/2	*Musculium kashmirensis*	China, Tibet, inflow Bangong Co	33.66933°	80.46586°	UGSB 11001	KF483378	KF483276	KF483351
CN19/3	*Musculium kashmirensis*	China, Tibet, inflow Bangong Co	33.66933°	80.46586°	UGSB 11002		KF483277	
CN20/1	*Pisidium stewarti*	China, Tibet, dam region near Bangong Co	33.56812°	79.94763°	UGSB 10991		KF483278	KF483353
CN20/2	*Pisidium stewarti*	China, Tibet, dam region near Bangong Co	33.56812°	79.94763°	UGSB 10992	KF483379	KF483279	
CN20/3	*Pisidium nitidum*	China, Tibet, dam region near Bangong Co	33.56812°	79.94763°	UGSB 10396		no sequence information	
CN21/1	*Pisidium subtruncatum*	China, Tibet, Bangong Co	33.57666°	79.86945°	UGSB 10993	KF483381	KF483281	KF483331
CN21/2	*Pisidium subtruncatum*	China, Tibet, Bangong Co	33.57666°	79.86945°	UGSB 10994	KF483380	KF483280	KF483332
CN21/3	*Pisidium* sp. I	China, Tibet, Bangong Co	33.57666°	79.86945°	UGSB 10995		KF483282	
CN21/4	*Pisidium nitidum*	China, Tibet, Bangong Co	33.57666°	79.86945°	UGSB 10999		KF483285	KF483354
CN21/5	*Pisidium nitidum*	China, Tibet, Bangong Co	33.57666°	79.86945°	UGSB 11000	KF483384	KF483286	
CN22/1	*Pisidium zugmayeri*	China, Tibet, wetlands north of Bangong Co	33.62711°	79.77509°	UGSB 10997	KF483382	KF483283	KF483333

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Spec. Code	Taxon	Locality	Latitude	Longitude	Spec. voucher #	COI Gen-Bank acc.#	16S Gen-Bank acc.#	28S Gen-Bank acc.#
CN22/2	*Pisidium zugmayeri*	China, Tibet, wetlands north of Bangong Co	33.62711°	79.77509°	UGSB 10998	KF483383	KF483284	
CN23	*Pisidium stewarti*	China, Tibet, ponds north of Coqen	31.07629°	84.99603°	UGSB 10230			
CN24/1	*Pisidium stewarti*	China, Tibet, estuary eastern inflow Bangong Co	33.55763°	79.93183°	UGSB 12596			
CN24/2	*Pisidium nitidum*	China, Tibet, estuary eastern inflow Bangong Co	33.55763°	79.93183°	UGSB 12597			
CN24/3	*Pisidium subtruncatum*	China, Tibet, estuary eastern inflow Bangong Co	33.55763°	79.93183°	UGSB 12598		no sequence information	
CN24/4	*Pisidium zugmayeri*	China, Tibet, estuary eastern inflow Bangong Co	33.55763°	79.93183°	UGSB 12599		no sequence information	
CN25	*Musculium kashmirensis*	China, Tibet, death channel south of Bangong Co	33.23835°	79.78357°	UGSB 10444		no sequence information	
CN26/1	*Pisidium stewarti*	China, Tibet, eastern inflow Bangong Co	33.55812°	80.05582°	UGSB 12600		no sequence information	
CN26/2	*Pisidium zugmayeri*	China, Tibet, eastern inflow Bangong Co	33.55812°	80.05582°	UGSB 12601		no sequence information	
CN27	*Musculium kashmirensis*	China, Tibet, southern inflow Bangong Co	33.41452°	79.64413°	UGSB 10488		no sequence information	
DE01/1^	*Pisidium nitidum*	Germany, Mecklenburg-Vorpommern	53.58138°	10.95250°	UGSB 10561	KF483385	EU559134	
DE02/1^	*Pisidium casertanum*	Germany, Brandenburg	52.24666°	14.45416°	UGSB 10562	KF483386	EU559092	
DE03/1	*Pisidium globulare*	Germany, Sachsen	51.33000°	14.48250°	UGSB 10563	KF483423		KF483334
DE04/1	*Pisidium supinum*	Germany, Brandenburg	52.48727°	13.64439°	UGSB 10564	KF483388		
DE05/1	*Pisidium obtusale*	Germany, Thüringen	51.08027°	10.48500°	UGSB 10565	KF483387		KF483335

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Spec. Code	Taxon	Locality	Latitude	Longitude	Spec. voucher #	COI Gen-Bank acc.#	16S Gen-Bank acc.#	28S Gen-Bank acc.#
GE01/1	*Pisidium personatum*	Georgia, Mzcheta-Mtianeti	42.07222°	44.95972°	UGSB 9045	KF483389	KF483288	KF483336
GE02/1	*Pisidium subtruncatum*	Georgia, Samegreto-Zemo Svaneti	42.23306°	41.84722°	UGSB 9047	KF483390	KF483287	KF483337
GE03/1	*Musculium lacustre*	Georgia, Gurien	42.04722°	41.76638°	UGSB 9048	KF483391	KF483289	
GR01/1	*Pisidium casertanum*	Greece, West Macedonia	40.90222°	21.82500°	UGSB 9075	KF483392	KF483290	KF483338
ID01/1	*Pisidium* sp.	Indonesia, Sulawesi	02.76075°	121.55860°	UGSB 10983		KF483291	KF483339
ID02/1	*Pisidium* cf. *dammermani*	Indonesia, Sulawesi	02.47667°	121.25977°	UGSB 10984	KF483393	KF483292	KF483340
IR01/1	*Pisidium casertanum*	Iran, Fars	29.57083°	51.94250°	UGSB 11142	KF483394	KF483293	KF483341
IT01/1	*Pisidium subtruncatum*	Italy, Caserta	41.09786°	14.33066°	UGSB 10566	KF483395	KF483294	
LS01/1	*Pisidium viridarium*	Lesotho, Thaba-Tseka	-29.51302°	28.52350°	UGSB 10567	KF483397	KF483296	
LS02/1	*Pisidium* cf. *casertanum*	Lesotho, Mokhotlong	-29.11645°	28.86351°	UGSB 10568	KF483396	KF483295	
MK01/1	*Pisidium henslowanum*	Macedonia, municipality of Resen	40.93665°	20.94414°	UGSB 10546	KF483398	KF483297	
MK02/1	*Pisidium maasseni*	Macedonia, municipality of Resen	40.87103°	20.98772°	UGSB 10549		KF483298	
MK03/1	*Pisidium* cf. *casertanum*	Macedonia, municipality of Struga	41.34999°	20.62245°	UGSB 10556	KF483399	KF483299	
MK04/1^	*Pisidium casertanum*	Macedonia, municipality of Struga	41.18526°	20.72862°	UGSB 10557	KF483400	EU559091	
MK05/1	*Pisidium personatum*	Macedonia, municipality of Resen	40.97319°	21.05432°	UGSB 10550	KF483401		
MK06/1^	*Pisidium subtruncatum* s. str.	Macedonia, municipality of Ohrid	40.91393°	20.74226°	UGSB 10559	KF483402	EU559153	
MK07/1	*Pisidium subtruncatum recalvum*	Macedonia, municipality of Ohrid	40.92063°	20.73580°	UGSB 10560		KF483300	
MK08/1	*Pisidium edlaueri*	Macedonia, municipality of Struga	41.09974°	20.63804°	UGSB 9058	KF483403	KF483301	KF483342
MK08/2	*Pisidium edlaueri*	Macedonia, municipality of Struga	41.09974°	20.63804°	UGSB 9059	KF483404	KF483302	
MW01/1	*Eupera* sp.	Malawi, Kassala	15.44374°	34.86392°	UGSB1224	KF483405	KF483303	
NA01/1	*Eupera* sp.	Namibia, Caprivi	-17.48629°	24.25615°	UGSB 5682		KF483304	

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Spec. Code	Taxon	Locality	Latitude	Longitude	Spec. voucher #	COI Gen-Bank acc.#	16S Gen-Bank acc.#	28S Gen-Bank acc.#
NO01/1	*Pisidium lilljeborgii*	Norway, Nordland	68.25166°	13.65750°	UGSB 10598	KF483406	KF483305	KF483343
NP01/1	*Pisidium kuiperi*	Nepal, Karnali	29.08333°	82.65000°	UGSB 10570	KF483407	KF483306	
NP02/1	*Pisidium atkinsonianum*	Nepal, Karnali	29.30000°	82.28333°	UGSB 10571	KF483408	KF483307	
NP03/1	*Musculium kashmirensis*	Nepal, Bagmati	27.59693°	85.37914°	UGSB 10573		KF483308	
NP03/2	*Pisidium annandalei*	Nepal, Bagmati	27.59693°	85.37914°	UGSB 9094	KF483409	KF483309	KF483330
NP03/3	*Pisidium kuiperi*	Nepal, Bagmati	27.59693°	85.37914°	UGSB 9097	KF483410	KF483310	KF483344
NP03/4	*Musculium indicum*	Nepal, Bagmati	27.59693°	85.37914°	UGSB 9098	KF483411	KF483311	KF483345
NP04/1	*Pisidium* sp.	Nepal, Mechi	27.51667°	87.70000°	UGSB 10577	KF483412	KF483312	KF483346
NP04/2	*Pisidium atkinsonianum*	Nepal, Mechi	27.51667°	87.70000°	UGSB 10594	KF483413	KF483313	
NP05/1	*Pisidium* sp.	Nepal, Mechi	27.48717°	87.74000°	UGSB 10583	KF483414	KF483314	
NP06/1	*Pisidium annandalei*	Nepal, Seti	29.47972°	81.12778°	UGSB 7004	KF483415	KF483315	
TR01/1	*Pisidium* cf. *nitidum*	Turkey, Isparta	37.88664°	30.85872°	UGSB 9064	KF483416	KF483317	KF483347
TR02/1	*Pisidium casertanum*	Turkey, Antalya	37.10960°	30.58551°	UGSB 9067	KF483418	KF483316	KF483348
TR02/2	*Pisidium personatum*	Turkey, Antalya	37.10960°	30.58551°	UGSB 9069	KF483417	KF483318	
UG01/1	*Sphaerium* sp.	Uganda, Mukono	0.22240°	32.63433°	UGSB 4856	KF483419	KF483319	KF483349
UG01/2	*Eupera ferruginea*	Uganda, Mukono	0.22240°	32.63433°	UGSB 4854	KF483420	KF483320	
UG02/1	*Eupera ferruginea*	Uganda, Buliisa	2.27527°	31.67599°	UGSB 5398	KF483421	KF483321	
VN01/1	*Pisidium nevillianum*	Vietnam, Ninh Bình	20.23361°	105.72194°	UGSB 10986		KF483322	KF483350
ZA01/1	*Pisidium costulosum*	South Africa, Mpumalanga	-27.01371°	29.87412°	UGSB 10599	KF483422	KF483323	

APPENDIX 2. List of additional 16S sequences obtained from GenBank including accession number, taxon, specimen code, locality information, and respective author(s).

16S Gen-Bank acc.#	Taxon	Spec. Code	Locality	Author(s)
AF152027	*Pisidium dubium*	US01/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152029	*Pisidium compressum*	US02/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152030	*Pisidium variabile*	US03/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152031	*Pisidium adamsi*	US04/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152032	*Pisidium sterkianum*	AR01/1*	Argentina, Buenos Aires	Cooley & Ó Foighil, 2000
AF152033	*Musculium securis*	US05/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152034	*Musculium argentinum*	AR01/2*	Argentina, Buenos Aires	Cooley & Ó Foighil, 2000
AF152035	*Musculium lacustre*	DE06/1*	Germany	Cooley & Ó Foighil, 2000
AF152036	*Musculium partumeium*	US06/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152037	*Sphaerium corneum*	DE07/1*	Germany	Cooley & Ó Foighil, 2000
AF152038	*Sphaerium rhomboideum*	US07/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152039	*Sphaerium fabale*	US08/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152040	*Sphaerium simile*	US09/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152045	*Sphaerium striatinum*	US10/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152046	*Sphaerium occidentale*	US11/1*	USA, Michigan	Cooley & Ó Foighil, 2000
AF152047	*Sphaerium novaezealandiae*	NZ01/1*	New Zealand	Cooley & Ó Foighil, 2000
AF152048	*Sphaerium tasmanicum*	AU01/1*	Australia, New South Wales	Cooley & Ó Foighil, 2000
AY093550	*Musculium japonicum*	JP01/1*	Japan, Ehime Prefecture	Lee & Ó Foighil, 2003
AY093552	*Musculium lacustre*	US12/1*	USA, Michigan	Lee & Ó Foighil, 2003
AY093553	*Musculium miyadii*	RZ01/1*	Russia, Kuril Islands	Lee & Ó Foighil, 2003
AY093554	*Musculium transversum*	US13/1*	USA, Michigan	Lee & Ó Foighil, 2003
AY093557	*Pisidium casertanum*	DE08/1*	Germany, Ammerbuch	Lee & Ó Foighil, 2003
AY093559	*Pisidium casertanum*	US14/1*	USA, Michigan	Lee & Ó Foighil, 2003
AY093562	*Pisidium hallae*	N/A.1*	N/A	Lee & Ó Foighil, 2003
AY093565	*Pisidium nipponense*	JP02/1*	Japan, Gunma Prefecture	Lee & Ó Foighil, 2003
AY093566	*Pisidium nitidum*	N/A.2*	N/A	Lee & Ó Foighil, 2003
AY093569	*Pisidium supinum*	N/A.3*	N/A	Lee & Ó Foighil, 2003
AY093570	*Pisidium parvum*	JP01/2*	Japan, Ehime Prefecture	Lee & Ó Foighil, 2003
AY093571	*Pisidium japonicum*	JP03/1*	Japan, Nagano Prefecture	Lee & Ó Foighil, 2003
AY093573	*Sphaerium nucleus*	UA01/1*	Ukraine	Lee & Ó Foighil, 2003
AY792320	*Sphaerium corneum*	LT01/1*	Lithuania, Vilnius	Petkeviciute et al., 2006
AY957824	*Pisidium fallax*	US15/1*	USA, Colorado	Guralnick, 2005
AY957832	*Pisidium hallae*	US16/1*	USA	Guralnick, 2005
AY957836	*Pisidium ventricosum*	US17/1*	USA, Colorado	Guralnick, 2005
AY957855	*Pisidium milium*	US18/1*	USA, Colorado	Guralnick, 2005
AY957858	*Pisidium nitidum*	US19/1*	USA, Colorado	Guralnick, 2005
AY957880	*Pisidium ferrugineum*	US20/1*	USA	Guralnick, 2005
AY957884	*Pisidium ferrugineum*	US21/1*	USA	Guralnick, 2005

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16S GenBank acc.#	Taxon	Spec. Code	Locality	Author(s)
DQ062627	*Pisidium moitesserianum*	N/A.4*	N/A	Steiner, 2006 (unpubl. data)
EU559087	*Pisidium amnicum*	AL02/1*	Albania, Tušemišta, Lake Ohrid	Schultheiss et al., 2008
EU559089	*Pisidium amnicum*	ME01/1*	Montenegro, Crnojevica River	Schultheiss et al., 2008
EU559090	*Pisidium annandalei*	IN01/1*	India, Pithoragarh	Schultheiss et al., 2008
EU559095	*Pisidium casertanum*	MK05/2*	Macedonia, Pretor, Lake Prespa	Schultheiss et al., 2008
EU559097	*Pisidium casertanum*	ES01/1*	Spain, Picos de Europa	Schultheiss et al., 2008
EU559104	*Pisidium clarkeanum*	NP07/1*	Nepal, Ghatgai w Sauraha	Schultheiss et al., 2008
EU559105	*Pisidium clarkeanum*	NP08/1*	Nepal, Haripur	Schultheiss et al., 2008
EU559110	*Pisidium edlaueri*	MK09/1*	Macedonia, Struga, Lake Ohrid	Schultheiss et al., 2008
EU559111	*Pisidium edlaueri*	MK10/1*	Macedonia, Ohrid town, Lake Ohrid	Schultheiss et al., 2008
EU559133	*Pisidium nevillianum*	NP09/1*	Nepal, Lamahi	Schultheiss et al., 2008
EU559147	*Pisidium pseudosphaerium*	DE03/2*	Germany, Uhyst	Schultheiss et al., 2008
EU559164	*Pisidium waldeni*	NO02/1*	Norway, Davggejavrre	Schultheiss et al., 2008